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What the distribution of sperm size can tell about the stability of spermatogenesis in hybrid frogs *Pelophylax esculentus*

A.O. Fedorova, E.S. Pustovalova

Interspecies hybrid frogs *Pelophylax esculentus* and one of its parental species *Pelophylax ridibundus* inhabit the Siversky Donets center of diversity of water frogs in Eastern Ukraine. These frogs can crossbreed and form progeny in population systems which are called hemiclonal (HPS). Such systems have their own exceptional features which make them interesting for studying. The Lower Dobrytskiy Pond, which is situated in the National Nature Park "Homilshansky lisy" and is a part of Siversky Donets river basin, is on focus. Current work is devoted to the combination of two methods of spermatogenesis investigation. First, using the method of Ag-staining we observed high variability of meiotic chromosomal plates in testes of 24 adult male water frogs *P. esculentus* (2n=26). Only one male had 100% of full meiotic plates with no aneuploid plates. A significant amount of studied males (21/24) produced aneuploid chromosomal plates (4-68% of the total amount of meiotic plates). This may lead to a decrease in their fertility or even to their entire sterility. Also, we have not observed any chromosomal meiotic plates in two of 24 males. Some males (8/24) even produced meiotic chromosomal plates with 26 bivalents (i.e. 4n germ cells) which may testify about the ability to produce diploid sperm. Further, the lengths of urinary sperm cells' heads were measured. Finally, we performed an analysis of both meiotic chromosomes in testes and the distribution of sizes of urinary sperm cells' heads of hybrid water frogs *Pelophylax esculentus* from Siversky Donets basin to find out if there is a link between these two features. No difference in sperm heads lengths was found between males producing moderate and low amounts of sperm. Based on the data of meiotic plates all males were assigned into five categories via PCA (principal component analysis). A significant difference in sperm heads lengths was found within the category I (males with mostly full meiotic plates). The analysed data shows that each male from the studied population has his own unique features. No direct link between sperm cells size and features of meiotic chromosomes in testes was found.

Key words: *Pelophylax, sperm, hybrid, chromosome, meiosis, spermatogenesis.*

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Introduction

Speciation is an adaptive process that can occur due to the accumulation of genetic diversity within individuals of one species (sympatric speciation). Long-term studies of hybrid organisms have shown that some hybrids can reproduce. Moreover, speciation by interspecific hybridization makes hybrids more evolutionarily successful (Dawley, Bogart, 1989; Schön et al., 2009; Neaves, Baumann, 2011; Stöck et al., 2021). The animals breeding by hybridogenesis include European water frogs belonging to several species complexes. The *Pelophylax esculentus* complex includes two parental species *Pelophylax lessonae* (Camerano, 1882) (LL-genotype) and *Pelophylax ridibundus* (Pallas, 1771) (RR-genotype) and their hybrid *Pelophylax esculentus* (Linnaeus, 1758) (LR-genotype). One of the parental species genomes is eliminated and another is duplicated during gametogenesis, which leads to the formation of identical germ cells and maintaining hemiclonal inheritance of a clonal hybrid frog genome (Heppich, et al., 1982). The existence of triploid forms (LRR- or LLR-genotype) in the water frog's complex allows the recombination of the clonal genome (Tunner, 1973; Graf, Polls Pelaz, 1989; Plötner, 2005).

Hybrid *P. esculentus* (di- and/or triploids) usually coexist with one or both parental species in hemiclonal population systems (Shabanov et al., 2020). To establish how different systems maintain their stability, it's necessary to investigate the gametogenesis of males and females. According to the previous studies (Ragghianti et al., 2007; Mykhailova et al., 2011; Dedukh et al., 2013, 2015, 2017; Vegerina et al., 2014; Biriuk et al., 2015, 2016), chromosomal plates with 13 bivalents (meiosis I) or 13 univalents (meiosis

II) are considered to be normal and potentially can form vital gametes. In studies on diploid males, it was shown that hybrids from the Siverskiy Donets River systems produce many aneuploid (less or more than 13 bi-/univalents) meiotic chromosomal plates (Mykhailovaet al., 2011; Vegerina et al., 2014; Biriuk et al., 2016). Also, they can produce either one type of gametes (L or R) or a mix of them (Vinogradov et al., 1990; Biriuk et al., 2016; Doležálková et al., 2016). However, unlike the females, adult males have a lot of disturbances in their testes (Dedukh et al., 2015, 2017; Biriuk et al., 2016; Doležálková et al., 2016). Additionally, it was shown that some hybrid frogs produced diploid gametes (Dedukh et al., 2015, 2017) and a double number of chromosomes in meiotic plates (26 bivalents instead of 13) (Ragghianti et al., 2007; Vegerina et al., 2014; Biriuk, 2017). The presence of meiotic chromosomal plates with 26 bivalents is a signal of premeiotic genome duplication and should result in the formation of diploid sperm. Since diploid sperm carries twice more amount of DNA, we assume that diploid sperm cells should have a larger size than the haploid ones. A significant difference in sizes between diploid and polyploid (tri-, tetraploid) sperm was shown for some "non-frog" species, for instance, loaches, oysters and koi carp (Dong et al., 2005; Yoshikawa et al., 2007; Wang et al., 2020). But none of those studies contains data on the analysis of gonadal meiotic chromosomes. The data on *P. esculentus* sperm analysis is scarce. Our previous results showed that there is no clear difference between sperm size of hybrid males (Stepanenko et al., 2017). However, that research had some disadvantages, for example, we did not analyze meiotic chromosomes distribution for these males.

In this paper we show the results of the investigation on one of the most interesting and studied systems in the Siverskiy Donets river basin, the system of the Dobrytskyi pond, where different ploidy and sex *P. esculentus* coexist with one of the parental species *P. ridibundus.* The presence of tetraploid juveniles was shown for this system (Shabanov et al., 2020), so based on this data, we suggested that both males and females can produce diploid gametes which leads to tetraploid progeny formation. Thus, diploid *P. esculentus* males from this population system are an interesting group for gametogenesis investigation. Karyological preparations on testes give us direct data on how spermatogenesis proceeds. However, this method requires sacrificing animals. On the other hand, we could indirectly refer to spermatogenesis characteristics by studying mature urinary sperm, which is an intravital and non-harmful method. Javanbakht and Fathinia (2020) studying *P. ridibundus* and *P. bedriagae* have shown the difference in sperm sizes between different populations. However, the method of sperm fixation they used, required sacrificing animals. Therefore, the aim of this work was to understand how the distribution of size and amount of urinary sperm corresponds with features of meiotic chromosomes in testes of adult male *P. esculentus.*

Materials and methods

We collected 24 adult male frogs in July 2016, 2017 and June 2020 at night using a torch in the Lower Dobrytskyi pond (Kharkiv region, Chuhuiv district, 49°37'40"N; 36°16'58"E). On the basis of morphological traits (Shabanov, 2015), frogs were identified as *P. esculentus*.

Measurements of sperm heads lengths were carried out on samples of urinary semen. Male frogs were injected with Surfagon-L (ТОВ "Ланс-Хім"), a synthetic analogue of gonadotropin-releasing hormone (Bobrova et al., 2014). Samples of semen were fixated with Carnoy's solution (3 methanol : 1 glacial acetic acid) and centrifuged (3000 rpm). The precipitate was mixed with 70% glacial acetic acid and dropped onto a slide heated to 60°C. During the fixation sperm cells usually lose tails and only sperm heads are further available for analysis (Fig 1).

First, frogs' ploidy was roughly estimated by measuring erythrocytes (Ogielska-Nowak, 1978; Bondarieva et al., 2012) and further confirmed by karyoanalysis (Vegerina et al., 2013; Biriuk, 2017).

After preparation of the sperm samples, frogs have been fed for 2 days and then injected with 0.1-0.3 ml of 0.05% colchicine. The next day, frogs were euthanized by continued exposure to ETAC vapour. We dissected the testes, intestine and bone marrow from each frog, hypotonized them with 0.07 KCl for 20 min, then fixed them three times with Carnoy's solution. We dropped each tissue cell suspension onto slides preheated to 60 °C and further stained them with $AgNO_3$ (Birstein, 1984). For each frog, we also measured snout-vent length (SVL) and testes length (Table 1).

We photographed dried slides with chromosomes and sperm under a light microscope (x100 objective, microscope Leica DM 2000, camera Leica DFC3000 G, LASX software), and counted meiotic chromosomal plates manually for each slide to estimate gametogenesis. Sperm heads lengths were measured with the program ToupView with the help of instruments "Line" and "Ark" for straight and curved

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sperm heads, respectively (Fig 1). For individuals with a moderate or high amount of sperm up to 100-150 cells were measured.

Fig. 1. Example of photo for measuring sperm cells' heads of *P. esculentus* males' urinary sperm. Tails were lost during the fixation process. Scale bar – 10 µm.

We analyzed 11 adult hybrid males *P. esculentus* with a blind method: AF analyzed spermatids' length, EP analyzed the distribution of chromosomal plates in testes in the same frogs simultaneously.

We divided individuals into groups using principal component analysis and then compared them using nonparametric tests.

Results and discussion

For each individual male, we analyzed meiotic chromosomal plates and counted the number of full (13 univalent or bivalent chromosomes), aneuploid plates (slightly less or more than 13) and plates with ±26 bivalents (Fig. 2). We did not analyze mitotic chromosomal plates, because it is impossible to distinguish between somatic and germline mitoses. In general, we analyzed 1362 meiotic chromosomal plates (~55 per frog) for 25 males.

Based on the data of meiotic chromosomes counts we performed principal component analysis and assigned all studied frogs into five categories (Fig. 3).

For males caught in summer 2020 and 2021, we also collected urinary sperm and measured lengths of its heads. Among 11 studied frogs, 10 had amounts of sperm sufficient for measuring (Table 1). One male did not have sperm at all in the sample. In general, we analyzed 962 sperm cells for 10 male *P. esculentus*.

To evaluate sperm productivity for each male, we roughly estimated the mean number of sperm cells in drops of sperm suspension (~50 µL) on slides. If the number of sperm was higher than 1000, such males were considered to have high productivity, those who had 100-1000, we marked as moderate, and <100 as low.

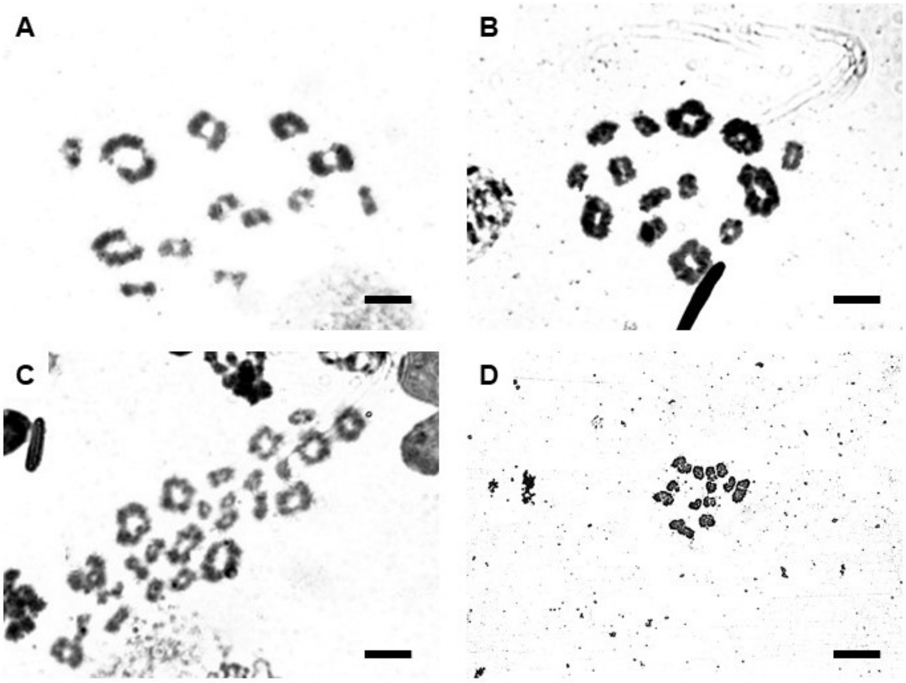

Fig. 2. Meiotic chromosomal plates in *P. esculentus*testes stained with $AgNO_3$. A - full plate with 13 bivalents, B – aneuploid plate, C – plate with 26 bivalents, D – highly condensed chromosomes of male №834 (see explanation in text). Scale bar – 10 µm.

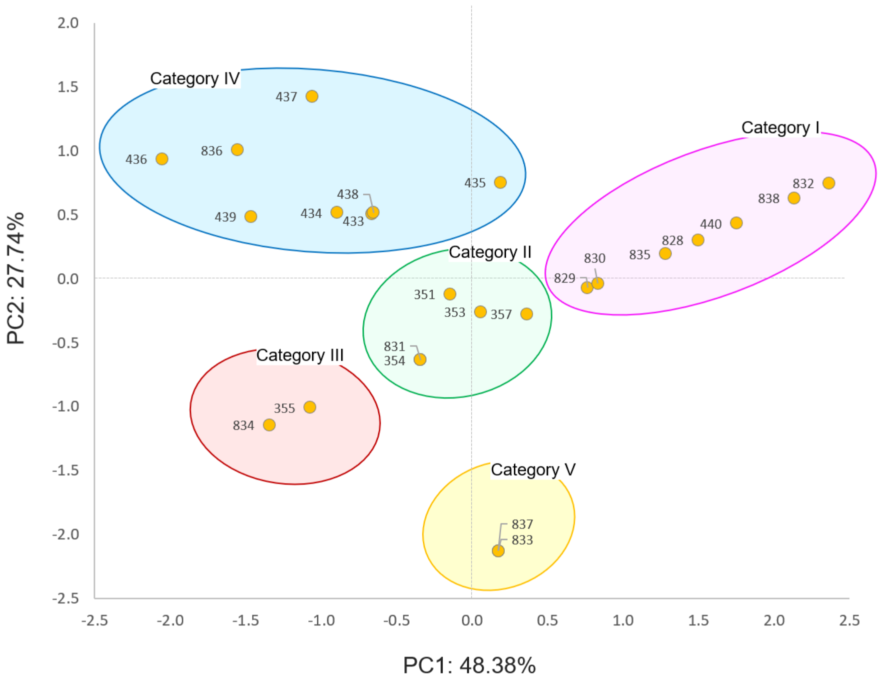

Fig. 3. Results of Principal component analysis. Category I (purple) - most plates have a full number of uni- or bivalents; category II (green) - the ratio of full and aneuploid plates is close to 1:1; category III (red) - most plates are aneuploid; category IV (blue) - a significant amount of plates with ±26 bivalents; category V (yellow) - no meioses were found.

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The shares of frogs assigned to each category differed between old (2016-2017) and new (2020) samples (p = 0.019, χ^2 = 6.925). Most of the males captured in 2020-2021 were assigned to Category I. Four of six males from category I had high or moderate amounts of sperm cells in urinary samples. Also, moderate amounts were detected in samples from males assigned to categories II, IV and V. Although category IV was characterized by the presence of plates with 26 bivalents, the only individual from this category, for whom we measured sperm size, had equal amounts of full and aneuploid meiotic plates, which is typical for category II.

The Kruskal-Wallis test showed the differences between groups of frogs with High, Moderate and Low sperm productivity (p < 0.0001). *Post hoc* analysis showed differences only in High-Moderate (p < 0.0001) and High-Low (p < 0.0001) pairs. Since only one male had high sperm productivity, we believe that the data is not enough to make any conclusions. However, no difference was found between groups “Moderate” and “Low” (p = 0.6582). We suggest that the number of urinary sperm produced by hybrid frogs at once has no connection to the length of sperm heads, but may be influenced by some other features, which require further investigation.

Table 1. Characteristics of all studied male *P. esculentus*.

№	Original sample ID	Ploidy	SVL*, mm	Testis, (left, right), mm	Full meiotic plates, %	Aneuploid meiotic plates, %	Meiotic plates with 26 bivalents, %	Relative sperm productivity
1	828	2n	73.5	5.6, 5.4	84	16	0	High
2	829	2n	73.5	5.7, 4.4	70	30	0	Moderate
3	830	2n	69.0	3.8, 3.6	72	28	0	Moderate
4	831	2n	71.0	4.7, 4.3	50	50	0	Moderate
5	838	2n	70.7	5.7, 5.3	96	4	0	Moderate
6	832	2n	75.1	5.6, 5.3	100	0	0	Low
7	834	2n	71.6	5.2, 5.1	32	68	0	Low
8	835	3n	67.4	4.4, 4.1	80	20	0	Low
9	836	2n	74.8	6.1, 3.2	49	51	11	Low
10	837	2n	70.4	4.6, 4.1	0	0	0	Low
11	833	2n	73.2	3.0, 3.0	0	0	0	No
12	351	2n	65.3	4.5, 3.1	59	41	3	-
13	353	2n	67.8	5.2, 4.5	59	41	1	-
14	354	3n	67.7	5.9, 4.5	50	50	0	-
15	355	2n	72.0	5.7, 5.6	37	63	0	-
16	357	2n	70.8	5.2, 4.1	63	37	0	-
17	433	2n	74.4	8.5, 5.9	61	39	8	-
18	434	2n	62.9	4.5, 4.5	58	42	9	-
19	435	2n	67.1	5.2, 5.2	74	26	7	-
20	436	2n	64.7	5.2, 5.2	46	54	17	-
21	437	2n	69.6	5.4, 4.3	67	33	17	-
22	438	2n	73.2	5.4, 4.5	60	40	8	-
23	439	2n	72.9	4.6, 3.6	48	52	11	-
24	440	2n	77.8	7.4, 6.5	89	11	0	-

**SVL - snout-vent length*

The only male that did not have sperm cells at all (№833), had only few chromosomal plates that were not countable, and very small testes (both 3.0 mm). The male №834 had a low amount of sperm and first, we didnot find any chromosomal plates while performing three consistent preparations from one of his testes. Further, we divided the other testis into 4 equal parts (segments were numbered randomly) and were able to count an efficient amount of meiotic chromosomal plates but did not find significant differences in amounts of plates in different segments ($p = 0.502$, $\chi^2 = 2.35$). The meiotic chromosomes of this individual were different from those we usually see in water frogs; they were highly condensed, round-shaped instead of elongated, and had no visual difference in size (Fig. 2D). High condensation and low amount of meiotic chromosomes corresponds with the absence of urinary sperm cells and indicate this male's sterility.

For each of the five categories of males, we analyzed the distribution of sperm cells sizes. Frogs from categories I and V have very similar distributions of sperm cells'heads lengths with one peak around 11 µm. Category II, in general, is characterized by smaller sperm cells'heads but with higher variability of lengths, whereas males from category III had slightly bigger sperm heads. Category IV was characterized by higher dispersion with a small predominance of cells about 12 µm. When analysing each individual frog from category I (Fig. 4B), three diploid males (№ 832, 830, 838) have a very similar size distribution while two more diploid males (№ 828, 829) and one triploid (№835) have noticeably larger or smaller sperm. The fact that triploid individual had a similar distribution of sperm cells size confirms the idea of similar spermatogenesis in di- and triploids (Christiansen, Reyer, 2009). We performed the Kruskal-Wallis test and found differences between males within this category ($p < 0.0001$).

We suggested that males which produced chromosomal plates with doubled number of chromosomes (26 bivalents, Fig. 2C) could form diploid gametes as was suggested by Biriuk O. V. (2017). In male №836 about 11% of chromosomal plates contained ±26 bivalents; the sperm cells lengths were not normally distributed (Shapiro-Wilk's W test, $p = 0.3047$, Fig. 4A). However, the median length of his sperm cells was comparable to the majority of others. Therefore, we cannot be confident that this male produced diploid gametes, this question requires further investigation.

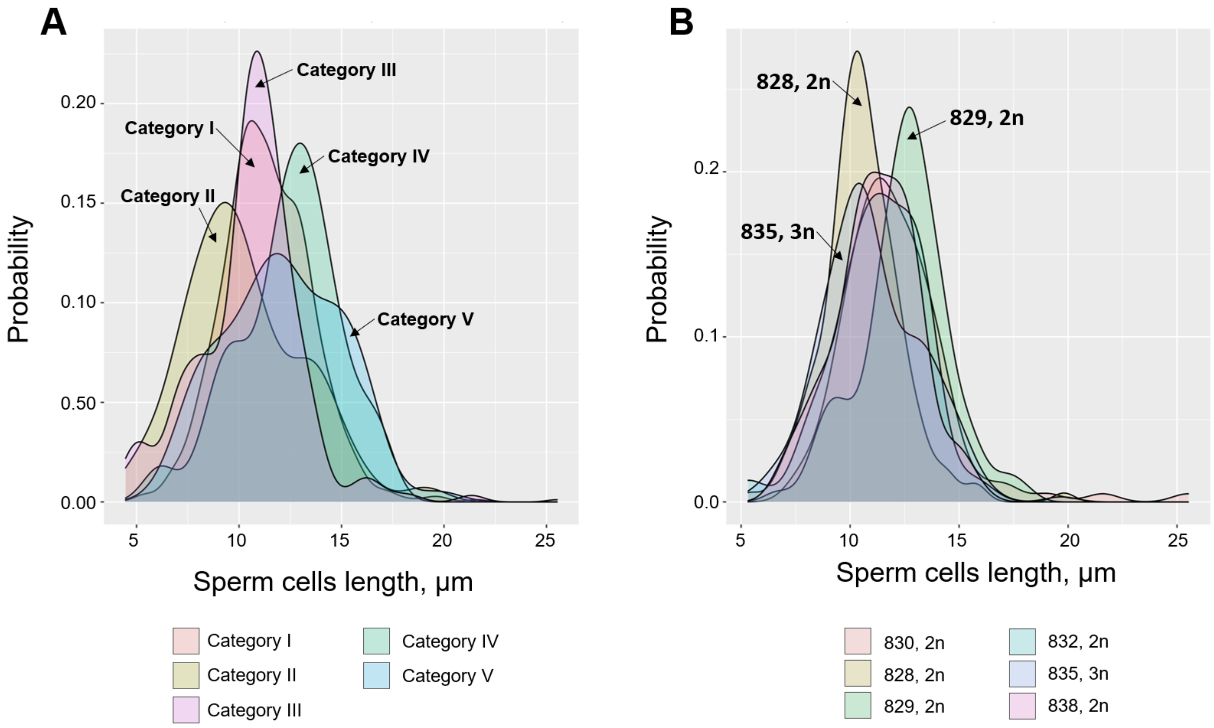

Fig. 4. Probability plots describing the distribution of sperm heads lengths in five categories (see fig. 3) of hybrid *P. esculentus*males (A) and for each individual male within category I (B).

Conclusions

Our data confirmed that each male from the studied population has his own unique features of spermatogenesis and sperm as well, which do not correspond to each other obviously. We did not find evidence that sperm size is connected to characteristics of meiotic chromosomes in testes. Probably, the size of sperm is determined by some other factors. Also, we did not observe "diploid" sperm cells produced

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by male from Category IV (26 bivalents). Sperm lengths certainly carry some information about the sperm formation process, but something other than karyotype variants in meiosis. In general, this indicates that the gametogenesis of interspecific hemiclonal hybrids, has many degrees of freedom, not all of which are clear to us at this stage of research.

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Що розподіл розмірів сперматозоїдів може розповісти про стабільність сперматогенезу у гібридних жаб *Pelophylax esculentus*

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Міжвидові гібриди *Pelophylax esculentus* та один з їх батьківських видів *Pelophylax ridibundus* населяють Сіверсько-Донецький центр різноманіття зелених жаб на Сході України. Вони можуть схрещуватися між собою і утворювати потомство в популяційних системах, які називаються геміклональними (ГПС). Такі системи мають свої виняткові особливості, які роблять їх цікавими для вивчення. У центрі уваги даної статті Нижній Добрицький став, який розташований в Національному природному парку «Гомільшанські ліси» і є частиною басейну річки Сіверський Донець. Дана робота присвячена поєднанню двох методів дослідження сперматогенезу. Спочатку за допомогою методу фарбування нітратом срібла ми спостерігали високу варіабельність мейотичних хромосомних пластинок у сім'яниках 25 дорослих самців зелених жаб *P. Esculentus* (2n=26). Тільки один самець мав 100% повних хромосомних мейотичних пластинок і жодної анеуплоїдної. Значна кількість досліджуваних самців (21 з 24) продукувала анеуплоїдні хромосомні пластинки (4-68% від загальної кількості). Це може бути ознакою зниження їх фертильності або взагалі призводити до повної стерильності. Також ми не зареєстрували жодної мейотичної хромосомної пластинки для двох з 24 самців. Деякі самці (8 з 24) навіть продукували мейотичні хромосомні пластинки з 26 бівалентами (тобто мали тетраплоїдних попередників статевих клітин). Далі ми вимірювали довжину сперматозоїдів, отриманих зі зразків уринальної сперми, і проводили аналіз мейотичних хромосом в сім'яниках та розподілу розмірів сперматозоїдів гібридів зелених жаб *P. esculentus* із басейну Сіверського Дінця, щоб з'ясувати, чи існує зв'язок між цими двома характеристиками. Різниця в довжині голівок сперматозоїдів між самцями, які продукували середню та низьку кількість сперматозоїдів, – незначуща. На основі даних мейотичних пластинок усіх самців розподілили на п'ять категорій за допомогою методу головних компонент. Значна різниця в довжині голівок сперматозоїдів була виявлена в межах першої категорії (самці з переважно повними мейотичними пластинками). Отримані дані показують, що кожен самець із досліджуваної популяції має свої унікальні особливості. Прямого зв'язку між розміром сперматозоїдів та особливостями мейотичних хромосом із сім'яників виявлено не було.

Ключові слова: *Pelophylax, сперматозоїди, гібрид, хромосома, мейоз, сперматогенез.*

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